Characterizing CXC Chemokine Ligand 12 (CXCL12) Conditional Knockout Mouse Model Systems

Research Thesis

Presented in partial fulfillment of the requirements for graduation with *research distinction* in the undergraduate colleges of The Ohio State University

by
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Abstract

CXC Chemokine Ligand 12 (CXCL12), also known as Stromal Derived Factor 1 (SDF-1), is the cognate ligand of CXC Chemokine Receptor Type 4 (CXCR4), a receptor that is highly expressed in human breast cancer. The CXCL12/CXCR4 signaling axis has been shown to play an important role in breast cancer progression and metastasis. It has been shown in previous studies that CXCL12 accelerates tumor growth and metastasis by modulating tumor stroma, an active element of the tumor microenvironment, via the increased development of carcinoma-associated fibroblasts (CAFs). CAFs, also known as myofibroblasts, are often observed in the stromal tissue of invasive breast cancers and promote angiogenesis through the recruitment of CXCR4-expressing endothelial precursor cells (EPCs). A better understanding of the CXCL12/CXCR4 signaling axis could result in the development of novel strategies to prevent breast cancer growth and metastasis.

CXCL12 conventional knockout mice have been previously generated. However, these mice are neonatally lethal, and are thus insufficient models for the characterization of breast cancer growth and metastasis. To circumvent this issue, our lab has begun the process of generating CXCL12 conditional knockout mice by inserting a CXCL12 targeting vector into C57BL/6 mice via blastocyst microinjection, resulting in the development of CXCL12 chimeric mice ($CXCL12^{loxPneo/+}$). The CXCL12 targeting vector inserted LoxP sequences 1 kb upstream of the start site of the CXCL12 gene, as well as in the second intron, flanking exons one and two of the CXCL12 gene. These $CXCL12^{loxPneo/+}$ chimeric mice were then crossbreed with C57BL/6 mice and the resulting $CXCL12^{loxPneo/+}$ non-chimeric progeny were characterized using PCR and Southern Blot analysis. A successful germline transmission was qualitatively indicated by an agouti coat color in the progeny.

In order to confirm the germline transmission of the CXCL12 targeting allele, the $CXCL12^{loxPneo/+}$ mice were breed with SRY-Box Containing Gene 2 – *Cre* Recombinase (Sox2-*Cre*) hemizygous mice, resulting in the *Cre* recombinase-mediated excision of the LoxP-flanked CXCL12 gene coding region throughout the early embryo. The neonatal

lethality observed in the resulting progeny indicated that the germline transmission of the CXCL12 targeting allele worked as intended, since neonatal lethality results from the deletion of the CXCL12 gene.

After confirming the germline transmission, the $CXCL12^{loxPneo/+}$ mice were breed with Flippase Reporter (FLPeR) mice to remove the Frt-flanked neomycin resistance (Neo) gene, which can influence the expression of the floxed CXCL12 gene. The removal of the Neo gene from the CXCL12 targeting allele resulted in the generation of $CXCL12^{loxP/+,\ FLPeR\ (+/-)}$ mice. These $CXCL12^{loxP/+,\ FLPeR\ (+/-)}$ mice were then interbreed for several generations to generate a sufficiently large population of $CXCL12^{loxP/loxP,\ FLPeR\ (-/-)}$ progeny; the target genotype of this research project.

In future studies, these $CXCL12^{loxP/loxP,\ FLPeR\ (-/-)}$ mice will be breed with Fibroblast Specific Protein 1 – *Cre* Recombinase (FSP-*Cre*) mice in order to delete the LoxP-flanked CXCL12 gene coding region from stromal fibroblasts in a cell/tissue-type specific manner. The deletion of the CXCL12 gene from stromal fibroblasts will result in the generation of neonatally viable CXCL12 conditional knockout mice. These CXCL12 conditional knockout mice will then be used to determine the role of the CXCL12/CXCR4 signaling axis in breast cancer growth and metastasis.

Introduction

Breast cancer is the most common form of invasive cancer in women in the United States and throughout the world. In the US, approximately 125 new cases of breast cancer are diagnosed each year per 100,000 women. Breast cancer is also the second leading cause of cancer death among women in the US, where there are approximately 23 deaths per 100,000 women annually.[3, 4, 6, 16]

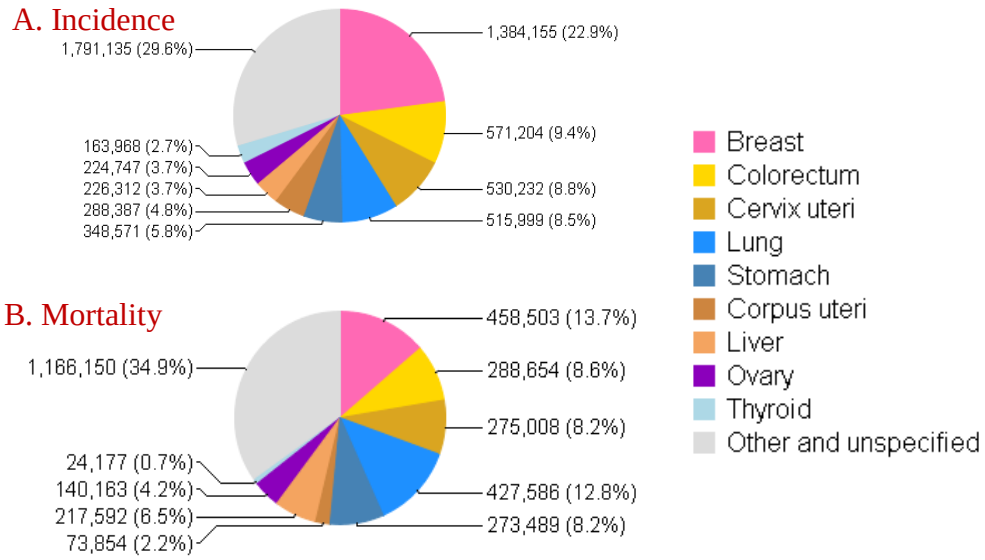


Figure 1. Breast Cancer Incidence and Mortality Statistics in Women Worldwide.[6] Breast cancer is the leading form of invasive cancer among women worldwide in both incidence and mortality rates. Non-invasive, non-melanomal skin cancers are included in these statistics under the 'Other and unspecified' categorization.

The prognosis and treatment options for breast cancer patients vary significantly depending on the specific classification of their breast cancer. This classification system categorizes breast cancers based on four major systems of grading: histopathology, grade, stage, and receptor status.

Common histological forms of breast cancer include Ductal Carcinoma *in situ* (DCIS), Lobular Carcinoma *in situ* (LCIS), Invasive Ductal Carcinoma (IDC), and Invasive Lobular Carcinoma (ILC).[2] Both DCIS and LCIS are non-invasive breast cancers that form within the milk ducts and lobules respectively, while IDC and ILC are both invasive breast cancers that metastasize into surrounding tissue.[17] Compared to non-invasive breast cancers, invasive breast cancers generally have less favorable prognoses due to

their ability to form secondary metastatic tumors. IDC is the most common histological form of breast cancer, accounting for approximately 55% of all breast cancers.[18] Chemokine receptor CXCR4, the cognate receptor of chemokine ligand CXCL12, has been shown to be expressed in 75% of IDC biopsy specimens.[12] Moreover, the over-expression of CXCR4 is correlated with a decreased survival rate in IDC patients.[12]

Breast cancer grade is categorized depending on the relative magnitude of differentiation of breast cancer cells, and includes three distinct grades. Grade 1 breast cancers are well-differentiated, grade 2 breast cancers are moderately differentiated, and grade 3 breast cancers are poorly differentiated.[2] Differentiation is defined as the development of cells into increasingly specialized forms, and is qualitatively measured by comparing the morphology of breast cancer cells with normal breast tissue cells. Breast cancer grade is generally determined using the Bloom–Richardson–Elston Grading System, also known as the Nottingham Grading System, which provides a comprehensive grade depending on three parameters: tubule formation, nuclear pleomorphism, and mitotic count.[19] Breast cancer prognoses become less favorable with a decreasing level of differentiation in breast cancer cells.

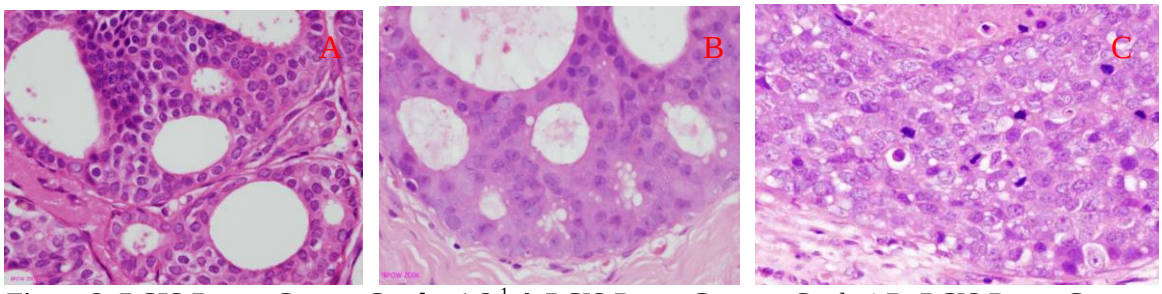


Figure 2. DCIS Breast Cancer Grades 1-3.[1] **A** DCIS Breast Cancer - Grade 1.**B**. DCIS Breast Cancer - Grade 2. **C**. DCIS Breast Cancer - Grade 3. Breast cancer cells become more irregular and less differentiated as the breast cancer grade increases.

Breast cancer stage is determined based on the TNM (Tumor, Nodes, Metastasis) Classification of Malignant Tumors, a cancer staging system which classifies a breast cancer's stage from 0 to IV based on three factors: the type of primary breast tumor, the size and location of breast cancer cells in various regional lymph nodes, and the prevalence of breast cancer metastasis.[20] The stage of a breast cancer contributes significantly to a patient's prognosis, ranging from a 92% five-year survival rate in stage

0 breast cancer patients to a 13% five-year survival rate in stage IV breast cancer patients.[21]

Breast Cancer Stage	**Prognosis (Five-Year Survival Rate)**
0	**92%**
I	**87%**
II	**75%**
III	**46%**
IV	**13%**

Table 1. Breast Cancer Stage vs. Prognosis.[21] Breast cancer prognoses become less favorable as the breast cancer stage increases.

Breast cancer receptor status classifies the various molecular subtypes of breast cancer based on the expression or non-expression of breast cancer associated receptors combined with breast cancer tumor grade. Important breast cancer associated receptors that influence the receptor status classification include the estrogen receptor (ER), the progesterone receptor (PR), and the Human Epidermal Growth Factor Receptor 2 (HER2/neu).[2] Different molecular subtypes of breast cancer have varying prognoses and recommended treatments. One common molecular subtype of breast cancer is Triple-Negative Breast Cancer (TNBC), which includes breast cancers that do not express the ER, PR, or HER2/neu genes.[22] It has been shown that poor clinical outcomes in TNBC patients are correlated with a high expression of CXCR4.[12]

Chemokines are a family of small cytokines, intercellular signaling proteins that are secreted by various types of cells. Chemokines possess the ability to induce chemotaxis, the directed movement of somatic cells in response to the presence of a particular molecule. Chemokines can either induce cell movement towards an area of higher chemokine concentration (positive chemotaxis) or lower chemokine concentration (negative chemotaxis).[23] Due to their ability to induce directed chemotaxis, chemokine signaling axes are involved in the progression and metastasis of several cancers, including breast cancer. There are 4 major chemokine types: CXC (α), CC (ß), C (γ), and CX_3C (δ), each differing in both structure and function.[11] CXCL12 is a type CXC

chemokine (α-chemokine), a type of chemokine that is composed of two N-terminal cysteine residues separated by a single amino acid residue.[11]

Chemokine receptors are G Protein-Coupled Receptors (GPCRs), a large family of G protein-linked receptors that are involved in signal transduction pathways in eukaryotes. The binding of a GPCR to its cognate ligand induces a conformational change in the GPCR's structure, which triggers the phosphorylation of guanosine diphosphate (GDP) to guanosine triphosphate (GTP). The phosphorylation of GDP induces the dissociation of the G Protein's α-subunit monomer and ßγ-subunit dimer from the G Protein-linked GPCR. The disassociated G protein subunits can then activate other molecules involved in the GPCR-associated signal transduction pathway.[12]

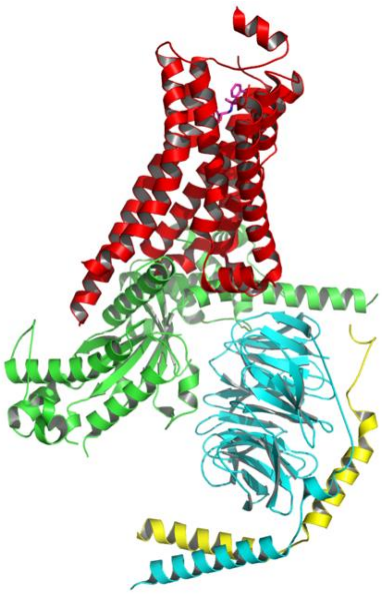


Figure 3. Crystal Structure of a GPCR –G Protein Complex.[24] **Red**: GPCR composed of 7 transmembrane domains. **Green**: G-Protein α-Subunit. **Blue**: G-Protein ß-Subunit. **Yellow**: G-Protein γ-Subunit.

CXCR4 is a type 4 α-chemokine receptor that binds to CXC chemokine ligands, specifically to CXCL12.[8] Similarly to other GPCRs, the binding of CXCR4 to its cognate ligand CXCL12 induces a conformational change in CXCR4's structure, which triggers the phosphorylation of GDP to GTP. The phosphorylation of GDP induces the dissociation of the α_i-subunit (G_α) and the ßγ-subunit complex ($G_{\beta\gamma}$) of the CXCR4-

coupled G protein, which both go on to modulate the downstream signaling of the CXCR4/CXCL12 signaling axis.[12]

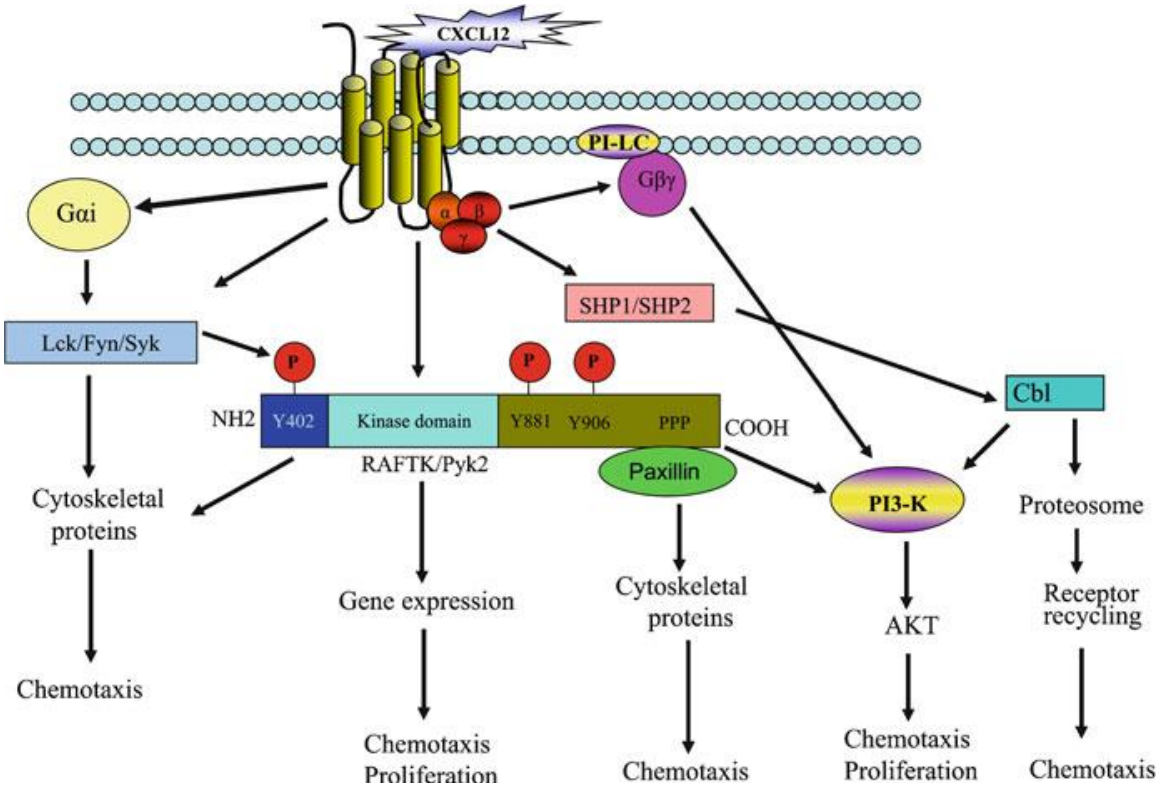


Figure 4. CXCL12/CXCR4 Signal Transduction Pathway.[12] This schematic diagrams the various signaling pathways in the CXCR4/CXCL12 signaling axis which result in the induction of chemotaxis and/or proliferation in human breast cancer.

These CXCR4-mediated, CXCL12-induced signaling pathways are shown in Figure 4. The CXCL12-induced chemotaxis of breast cancer cells is facilitated by intermediary genes and proteins, which are activated by the disassociated components of the CXCR4-coupled G protein (G_{α} and $G_{\beta\gamma}$). This CXCL12-induced chemotaxis of breast cancer cells can result in breast cancer proliferation and metastasis.[12]

The CXCR4/CXCL12 signaling axis has been shown to mediate organ-selective metastasis in breast cancers. Metastatic breast cancer cells were shown to over-express CXCR4, while metastasis-susceptible organs (lungs, bones, lymph nodes) were shown to over-express CXCL12. These observations indicate that CXCR4 and CXCL12 are involved with the trafficking of metastatic breast cancer cells.[12]

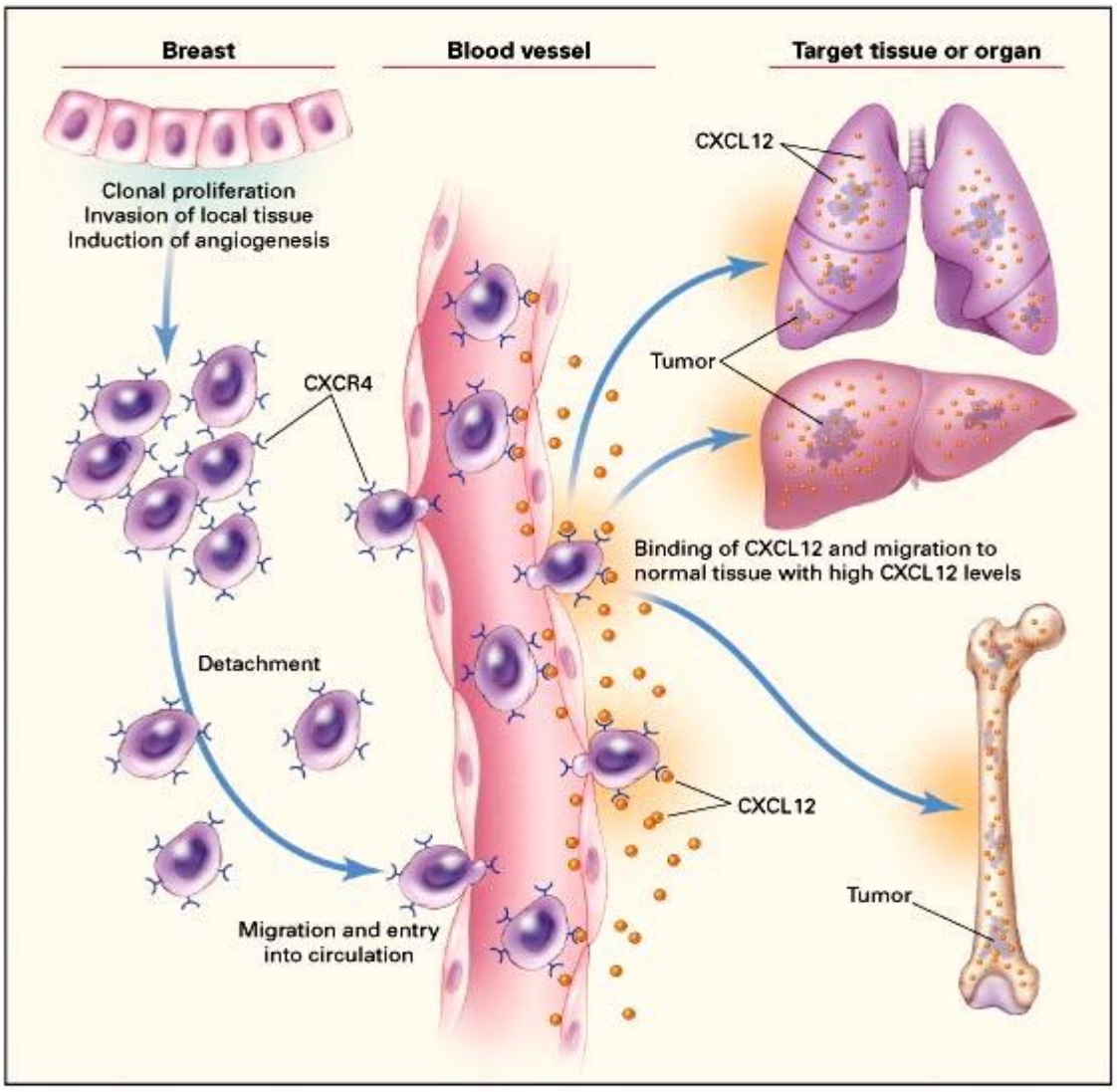


Figure 5. CXCL12/CXCR4 Regulation of Organ-Selective Breast Cancer Metastasis.[12] This model diagrams the CXCR4-mediated, CXCL12-induced organ-selective metastasis of breast cancer cells.

As depicted in Figure 5, cancerous mammary epithelial cells initially undergo clonal proliferation and invade the local breast tissue. After inducing angiogenesis, the breast cancer cells that express CXCR4 detach from the primary tumor and enter the blood stream after migrating across the lymphatic and vascular tumor walls. The CXCR4-expressing breast tumor cells then attach to organs (lungs, bones, lymph nodes) with vascular endothelial cells that express high levels of CXCL12 on the surface. The binding of CXCR4 to CXCL12 allows the CXCR4-expressing breast tumor cells to migrate into the organ tissue, where they can then proliferate, induce angiogenesis and form secondary metastatic tumors.[12]

The CXCL12/CXCR4 signaling axis has also been shown to regulate tumor growth and metastasis in breast cancers by modulating tumor stroma, an active element of the tumor microenvironment.[12]

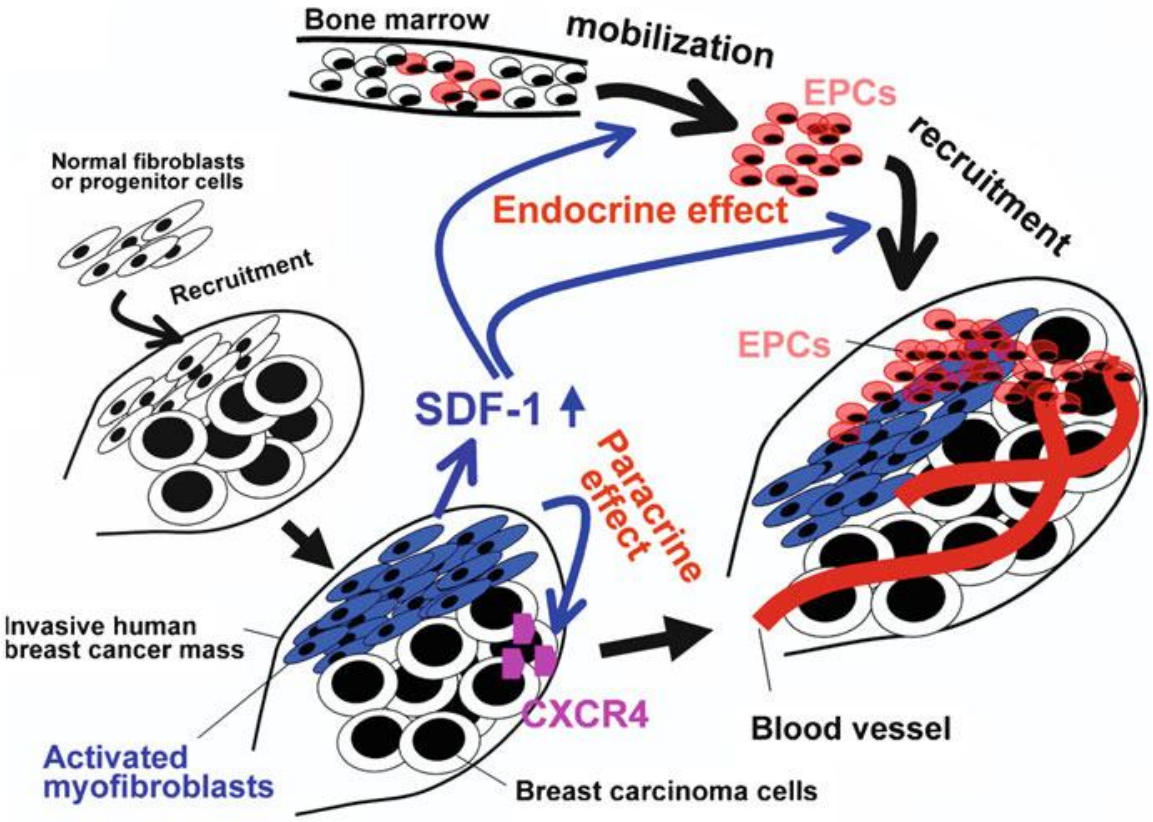


Figure 6. CXCL12-Induced Regulation of Breast Cancer Metastasis.[12] The CXCL12 released by stromal fibroblasts promotes tumorigenesis via two different mechanisms: the stimulation of angiogenesis through the recruitment of EPCs (endocrine effect), and the direct stimulation of breast tumor cells through CXCR4-expressing breast carcinoma cells (paracrine effect).

The tumor stroma consists of breast tumor cells, fibroblasts, endothelial cells, immune cells, and the extracellular matrix (ECM). The CXCL12/CXCR4 signaling axis has been shown to accelerate breast cancer growth and metastasis via the increased development of carcinoma-associated fibroblasts (CAFs). CAFs are often observed in the stromal tissue of invasive breast cancers and promote angiogenesis through the production of ECM as well as the secretion of growth factors and chemokines. As shown in Figure 6, CAFs that secrete CXCL12 have been shown to stimulate angiogenesis via the recruitment of CXCR4-expressing endothelial precursor cells (EPCs) to the tumor stroma.[12]

The CXCR4/CXCL12 signaling axis has been shown to play a role in breast cancer tumor proliferation, angiogenesis, and migration, and metastasis. The generation of CXCL12 conditional knockout mice will allow our lab to determine the role of the CXCR4/CXCL12 signaling axis in breast cancer growth and metastasis in *in vivo* mouse model systems. Since mouse model systems can approximate human model systems, the determination of the role of CXCR4/CXCL12 signaling axis can eventually lead to the development of novel strategies in the treatment of human breast cancer.

Methods

Generation of CXCL12$^{loxPneo/+}$ Chimeric Mice

The CXCL12 gene, located on mouse chromosome 6, encodes for 3 isoforms of CXCL12: α, ß, and γ. The coding region for CXCL12α contains 3 exons, while the coding region for CXCL12ß and CXCL12γ contain 4 exons. In order to knockout all 3 isoforms of CXCL12, LoxP sequences were inserted 1 kbp upstream of the start site of the CXCL12 gene and in the second intron using a CXCL12 targeting vector. Upon the introduction of *Cre* recombinase with FSP-*Cre* mice, the LoxP-flanked region will be deleted from stromal fibroblasts in a cell/tissue-type specific manner via the removal of the start site and promoter region of the CXCL12 coding region, resulting in the ablation of all isoforms of CXCL12 from stromal fibroblasts.

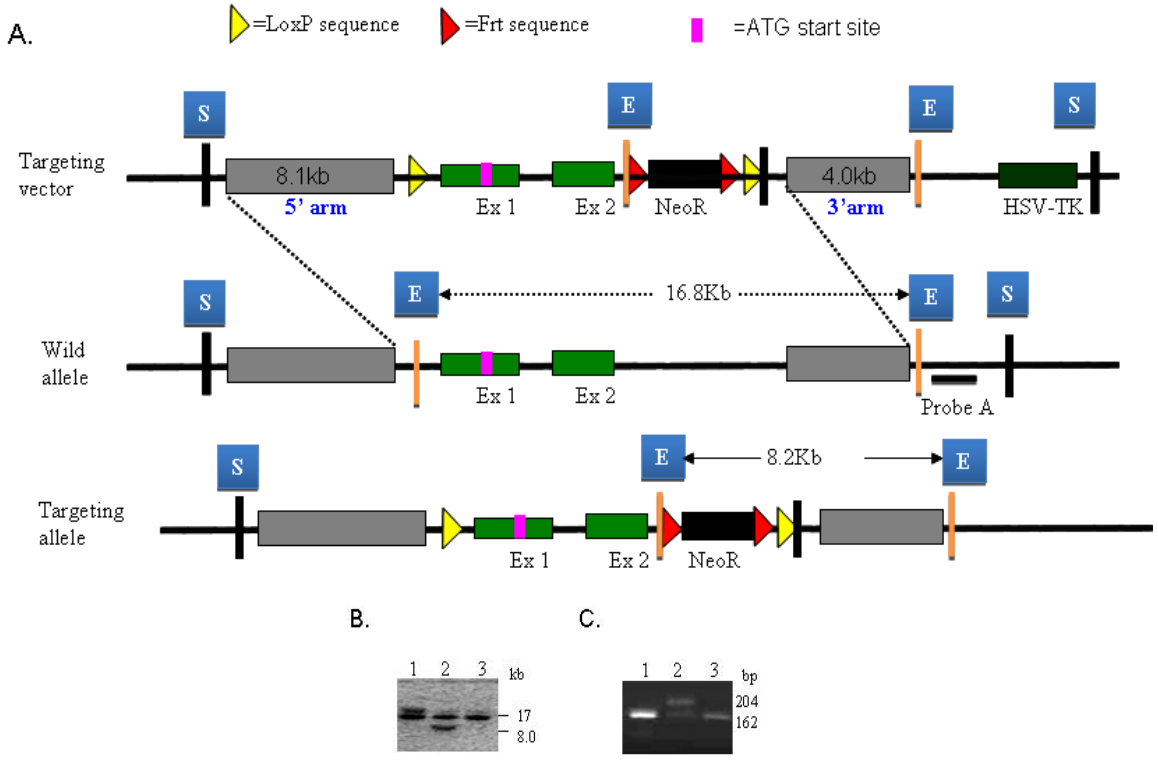


Figure 7. A. CXCL12 Gene Construct. This schematic diagrams the CXCL12 targeting vector, the CXCL12 wildtype allele, and the CXCL12 targeting allele. The CXCL12 targeting vector will insert LoxP sequences flanking the first two exons of the CXCL12 wildtype allele, as well as a Frt-flanked neomycin resistance gene, resulting in the generation of the CXCL12 targeting allele. **B. Southern Blot Identification of Positive CXCL12 BAC Clones**. 1. Wildtype; 2. CXCL12 Targeting Allele (CXCL12 Positive ES Clone); 3. CXCL12 Wildtype Allele. **C. PCR Analysis of LoxP$_1$ Sequence**. 1. Wildtype; 2. CXCL12 Targeting Allele (CXCL12 Positive ES Clone); 3. CXCL12 Wildtype Allele.

To create a CXCL12 targeting vector, as depicted in Figure 7-A, fragments of the CXCL12α gene were amplified from the genomic DNA of a 129/SvEvTac mouse strain and confirmed via sequencing. The sequenced CXCL12α gene fragments were then cloned using a bacterial artificial chromosome (BAC) DNA construct. Southern blot assays were used to identify BAC clones positive for CXCL12. A CXCL12 targeting vector was then created by recombineering the CXCL12 DNA from BAC clones into PL253 and PL451 vectors. The final vector, consisting of a long 5' arm (8.1 kbp), a short 3' arm (4.0 kbp), and a LoxP-flanked region (7.0 kbp), was linearized with *Sal*1, a high-fidelity restriction endonuclease, and transfected into TCl embryonic stem (ES) cells via electroporation. Successful transfection was coupled with the development of neomycin resistance in the transfected ES cells. The successfully transfected ES cells were selected via treatment with neomycin, and transfection was confirmed using southern blot and PCR analysis.

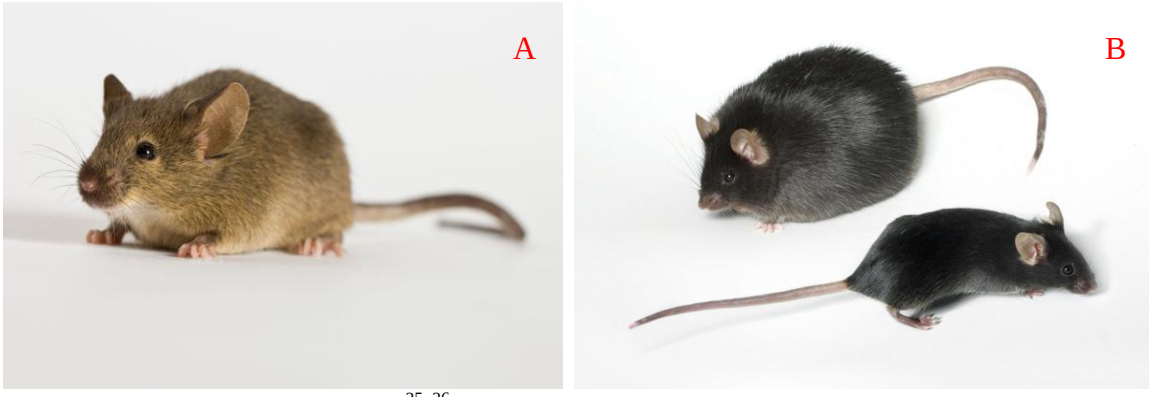


Figure 8. Agouti vs. C57BL/6 Mice.[25, 26] This figure depicts examples of **A.** Agouti mice and **B.** C57BL/6 mice. Agouti mice have a distinctive orange coat color that allows for a clear differentiation between agouti and C57BL/6 mice.

Confirmed embryonic stem cell clones were then microinjected into C57BL/6 blastocysts to generate male CXCL12$^{loxPneo/+}$ chimeric mice. The male CXCL12$^{loxPneo/+}$ chimeras were breed with female C57BL/6 wildtype mice to generate CXCL12$^{loxPneo/+}$ non-chimeric mice. Successful germline transmission was qualitatively indicated by 99.9% agouti characteristics in the resulting progeny.

Distal Tail Biopsy

The distal tail biopsy of CXCL12 mice was performed following the guidelines provided by the Institutional Animal Care and Use Committee (IACUC), an organization that oversees and ensures the humane treatment of animals which are used in scientific research. The IACUC guidelines facilitate the maximization of information derived from every animal used in scientific research, while simultaneously minimizing procedures that could cause discomfort, distress, or pain to laboratory animals.[9] These guidelines are enforced by University Laboratory Animal Resources (ULAR) in almost all *in vivo* biomedical research performed at The Ohio State University.

In order to obtain DNA for the genetic characterization of CXCL12 mice, a distal tail biopsy was performed on each CXCL12 mouse. Prior to the excision of the distal tail, each mouse's right ear was tagged using a Stainless Steel Applicator to apply a 4-digit numbered tag, in order to facilitate the identification of laboratory mice. Using a sterile pair of surgical scissors, ~5 mm of the distal tail was excised from each mouse prior to reaching 21 days of age. Since each distal tail biopsy was performed before each mouse reached 21 days of age, prior to the formation of calcified, ossified, and innervated vertebrae within the distal 5 mm of the tail, the use of a general anesthetic was not required.[9] After the excision of the distal tail, Kwik-Stop styptic powder with benzocaine, an anti-hemorrhagic/hemostatic agent combined with a topical anesthetic, was applied to the point of excision in order to promote hemostasis.

DNA Extraction, Isolation, and Purification

The DNA extraction of excised CXCL12 mouse distal tail tips was performed using the Promega Wizard® Genomic DNA Purification Kit.[10] A solution consisting of a 25:6 ratio of Nuclei Lysis Solution and 0.5 M Ethylenediaminetetraacetic Acid (EDTA) was chilled on ice until it turned cloudy/opaque. To each excised tail tip, 600 µL of the pre-chilled EDTA/Nuclei Lysis Solution was added to facilitate the lysis of nuclei from mouse tail tissue. Afterwards, 17.5 µL of 20 mg/mL Proteinase K, a broad spectrum serine protease, was added to each tail sample to induce proteolysis. Each sample was then incubated

overnight in a 55º C water bath with gentle shaking until the tail tissue was completely dissolved in the lysis solution.

After the overnight incubation, 3 µL of 4 mg/mL RNase A, a ribonuclease that catalyzes the degradation of single-stranded RNA (ssRNA), was added to each nuclear lysate. Each nuclear lysate sample was incubated at 37ºC for 30 min and then cooled to room temperature for 5 min. To each room temperature sample, 200 µL Protein Precipitation Solution was added, and the resulting solution was vortexed for 20 s and chilled on ice for 5 min. The chilled sample was centrifuged at 14,000 rpm for 4 min, yielding a pellet containing the precipitated protein and a supernatant containing the nuclear DNA. To prevent the transfer of waste material, the supernatant was immediately transferred to a sterile 1.5 mL microcentrifuge tube containing 600 µL room temperature isopropanol. The resulting solution was gently mixed via inversion until the white thread-like strands of isolated nuclear DNA formed a visible mass. The solution was then centrifuged at 14,000 rpm for 1 min, yielding a white DNA pellet after decanting the supernatant. To each DNA pellet, 600 µL of room-temperature 70% ethanol was added, and the resulting solution was gently mixed via inversion to remove hydrophobic contaminants. The mixed solution was then centrifuged at 14,000 rpm for 1 min, yielding a white DNA pellet. The supernatant was aspirated using a micropipet, and the purified DNA pellet was allowed to air-dry for 20 min to remove any remaining traces of 70% ethanol. To each air-dried DNA sample, 200 µL of DNA Rehydration Solution was added, and the resulting solution was incubated at 4º C overnight to facilitate the solubilization of the purified DNA.

Polymerase Chain Reaction (PCR)

PCR was used to genetically characterize CXCL12 mice via the use of gene-specific primers. The Sigma AccuTaq™ LA DNA Polymerase Kit was used to amplify the DNA isolated from the excised distal tail tips of CXCL12 mice.[13] Each CXCL12 mouse was genetically characterized by amplifying various combinations of the following gene fragments.

$LoxP_1$

To a 1.5 mL microcentrifuge tube, 1.25 µL AccuTaq LA 10x Buffer, 0.625 µL 10 mM dNTP mix, 0.25 µL Dimethyl Sulfoxide (DMSO), 0.25 µL 10 pmol/µL $LoxP_1$-F Primer, 0.25 µL 10 pmol/µL $LoxP_1$-R Primer, 0.125 µL 5 units/µL AccuTaq LA DNA Polymerase, and 9.25 µL Nuclease-Free H_2O was added per sample, yielding a Master Mix Solution. All primers were dissolved in TE Buffer. After the Master Mix Solution was homogenized via centrifugation at 5,000 rpm for 1 min, 12.25 µL was transferred to pre-labeled 200 µL PCR tubes. To each PCR tube, 0.25 µL of the previously isolated genomic DNA template was added, corresponding to the CXCL12 mouse it was isolated from. A Positive Control was created via the addition of 0.25 µL of a $LoxP_1$-homozygous DNA template in order to mark the expected size of the PCR product. Additionally, a Negative Control was created via the addition of 0.25 µL Nuclease-Free H_2O in order to determine the presence of DNA template contaminants in any of the components used in the master mix solution. Each PCR sample was centrifuged at 5,000 rpm for 1 min prior to being amplified in a Thermal Cycler using the following cycling parameters:

Step	Temperature	Incubation Time per Cycle	Number of Cycles
Initial Denaturation	98 ºC	30 s	×1
Denaturation	94 ºC	15 s	×35
Annealing	59 ºC	30 s	
Extension	68 ºC	120 s	
Final Extension	68 ºC	180 s	×1
Hold	4 ºC	Indefinite	×1

$LoxP_2$

To amplify $LoxP_2$, the procedure used to amplify $LoxP_1$ was repeated with the following adjustments:

1. $LoxP_2$-F Primer was used in place of $LoxP_1$-F Primer.
2. $LoxP_2$-R Primer was used in place of $LoxP_1$-R Primer
3. A $LoxP_2$-positive DNA template was used to make the positive control instead of a $LoxP_1$-homozygous DNA template.
4. The following thermal cycling parameters were used:

Step	Temperature	Incubation Time per Cycle	Number of Cycles
Initial Denaturation	98 ºC	30 s	×1
Denaturation	94 ºC	15 s	×35
Annealing	58 ºC	30 s	
Extension	68 ºC	120 s	
Final Extension	68 ºC	180 s	×1
Hold	4 ºC	Indefinite	×1

$LoxP_1/LoxP_2$

To amplify $LoxP_1/LoxP_2$, the procedure used to amplify $LoxP_1$ was repeated with the following adjustments:

1. $LoxP_2$-R Primer was used in place of $LoxP_1$-R Primer

2. A $LoxP_1/LoxP_2$,-positive DNA template was used to make the positive control instead of a $LoxP_1$-homozygous DNA template.

3. The following thermal cycling parameters were used:

Step	Temperature	Incubation Time per Cycle	Number of Cycles
Initial Denaturation	98 ºC	30 s	×1
Denaturation	94 ºC	15 s	×35
Annealing	58 ºC	30 s	
Extension	68 ºC	180 s	
Final Extension	68 ºC	300 s	×1
Hold	4 ºC	Indefinite	×1

LoxP/Neo

To amplify a combination of LoxP and Neo, the procedure used to amplify $LoxP_1$ was repeated with the following adjustments:

1. Frt_1-F Primer was used in place of $LoxP_1$-F Primer.

2. $LoxP_2$-R Primer was used in place of $LoxP_1$-R Primer.

3. 0.25 µL 10 pmol/µL Neo-F Primer was added per sample to the Master Mix Solution.

4. 9.00 µL Nuclease-Free H_2O was added per sample to the Master Mix Solution instead of 9.25 µL Nuclease-Free H_2O.

5. A LoxP-homozygous/Neo-positive DNA template was used to make the positive control instead of a $LoxP_1$-homozygous DNA template.

6. The following thermal cycling parameters were used:

Step	Temperature	Incubation Time per Cycle	Number of Cycles
Initial Denaturation	98 ºC	30 s	×1
Denaturation	94 ºC	15 s	×35
Annealing	58 ºC	30 s	
Extension	68 ºC	180 s	
Final Extension	68 ºC	600 s	×1
Hold	4 ºC	Indefinite	×1

FLPeR

To amplify the FLPeR gene, the procedure used to amplify $LoxP_1$ was repeated with the following adjustments:

1. IMR 1348 Primer was used in place of $LoxP_1$-F Primer.

2. IMR 1349 Primer was used in place of $LoxP_1$-R Primer.

3. A FLPeR-positive DNA template was used to make the positive control instead of a $LoxP_1$-homozygous DNA template.

4. The following thermal cycling parameters were used:

Step	Temperature	Incubation Time per Cycle	Number of Cycles
Initial Denaturation	98 ºC	30 s	×1
Denaturation	94 ºC	15 s	×35
Annealing	58 ºC	50 s	
Extension	68 ºC	90 s	
Final Extension	68 ºC	420 s	×1
Hold	4 ºC	Indefinite	×1

Sox2-*Cre*

To amplify the Sox2-*Cre* gene, the procedure used to amplify $LoxP_1$ was repeated with the following adjustments:

1. *Cre*-3 Primer was used in place of $LoxP_1$-F Primer.

2. *Cre*-1 Primer was used in place of $LoxP_1$-R Primer

3. A Sox2-*Cre*-positive DNA template was used to make the positive control instead of a $LoxP_1$-homozygous DNA template.

4. The following thermal cycling parameters were used:

Step	Temperature	Incubation Time per Cycle	Number of Cycles
Initial Denaturation	98 ºC	30 s	×1
Denaturation	94 ºC	15 s	×35
Annealing	55 ºC	30 s	
Extension	68 ºC	45 s	
Final Extension	68 ºC	180 s	×1
Hold	4 ºC	Indefinite	×1

Agarose Gel Electrophoresis

After thermal cycling was completed, 1.25 µL10x Gel Loading Buffer was added to each amplified DNA sample in order to facilitate the loading and tracking of the PCR samples. $LoxP_1$, $LoxP_2$, $LoxP_1/LoxP_2$, and Sox2-*Cre* PCR products were run on a 2.0% agarose gel, while LoxP/Neo and FLPeR PCR products were run on a 1.0% agarose gel. Each agarose gel was initially run at 100 V for 5 min, before running at 150 V until completion. Each gel was then imaged using a BIO-RAD ChemiDoc XRS Gel Scanner using the ultraviolet (UV) transillumination filter with the automatic exposure setting.

Materials

Distal Tail Biopsy

ARC Laboratories Kwik Stop® Styptic Powder with Benzocaine

National Band & Tag Company Stainless Steel Applicator

Surgical Scissors

DNA Extraction, Isolation, and Purification

Fischer Scientific 1.5 mL Microcentrifuge Tubes

Promega Nuclei Lysis Solution: Proprietary Ingredients

0.5 M EDTA: 0.5 M EDTA in ddH_2O

Invitrogen Proteinase K (Fungal): 20 mg/mL Proteinase K (20 units/mg) in 50 mM Tris-HCl (pH 8.0), 3 mM $CaCl_2$, 50% glycerol

Promega RNase A: 4 mg/mL RNase A in 10 mM Tris-HCl (pH 7.4), 1 mM EDTA (pH 8.0)

Promega Protein Precipitation Solution: Proprietary Ingredients

100% Isopropanol

70% Ethanol

Promega DNA Rehydration Solution: 10 mM Tris-HCl (pH 7.4), 1 mM EDTA (pH 8.0)

Polymerase Chain Reaction (PCR)

BIO-RAD C1000™ Thermal Cycler

Fischer Scientific 1.5 mL Microcentrifuge Tubes

Thermo Scientific PCR Tubes: 200 µL Thin-Walled Tubes with Flat Caps

Sigma AccuTaq™ LA 10x Buffer: 500 mM Tris-HCl, 150 mM $(NH_4)_2SO_4$ (pH 9.3), 25 mM $MgCl_2$, 1% polysorbate 20 (TWEEN® 20)

Thermo Scientific Fermentas dNTP Mix: 10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dTTP in Nuclease-Free H_2O

Sigma Dimethyl Sulfoxide: 100% Dimethyl Sulfoxide (DMSO)

Sigma AccuTaq™ LA DNA Polymerase: 5 units/µL DNA Polymerase in 20 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5% octylphenoxypolyethoxyethanol (IGEPAL®), 50% glycerol

Ambion DEPC-Treated Nuclease-Free Water: 0.2 µm Filtered, autoclaved

Invitrogen Tris-EDTA (TE) Buffer

Sigma Primers: 10 pmol/µLPrimer in TE Buffer

Primer Sequences	
Primer	**Sequence (5' → 3')**
$LoxP_1$-F	GCTTTGCTCTTAGCCTGCAC
$LoxP_1$-R	CCAGATGCTCCTAGGCAAAA
$LoxP_2$-F	TGCTATACGAAGTTATTAGGTGGA
$LoxP_2$-R	CAGGACACATCTCTGCCAAG
Frt_1-F	CACTCTTGCTTTCCAAGCTGT
NEO-F	ATAGCAGCTTTGCTCCTTCG
IMR 1348	CACTGATATTGTAAGTAGTTTGC
IMR 1349	CTAGTGCGAAGTAGTGATCAGG
Cre-3	ATGCTTCTGTCCGTTTGCCG
Cre-1	CCTGTTTTGCACGTTCACCG

Agarose Gel Electrophoresis

BIO-RAD ChemiDoc XRS Gel Scanner

Invitrogen BlueJuice™ 10x Gel Loading Buffer: 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, 0.3% Bromophenol Blue, 65% Sucrose

Invitrogen UltraPure™ 10x TBE Buffer

Invitrogen Ethidium Bromide (EtBr): 10 mg/mL EtBr

2.0% Agarose Gel: 2.0% Agarose in 0.5x TBE Buffer, 0.50 mg/mL EtBr

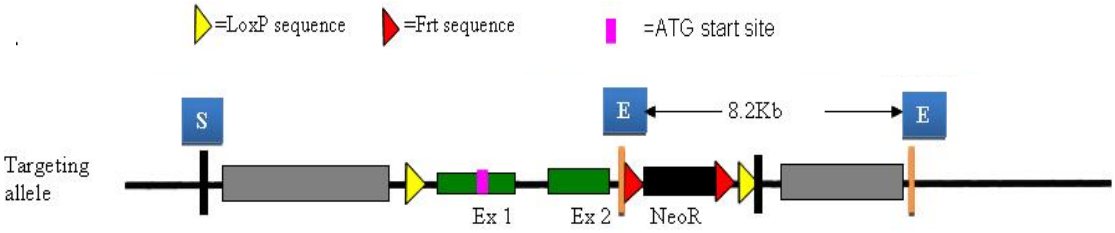
1.0% Agarose Gel: 1.0% Agarose in 0.5x TBE Buffer, 0.50 mg/mL EtBr

Results

Sox2-*Cre*–Mediated Deletion of CXCL12

A breeding scheme was constructed in order to confirm the location of the LoxP sequences inserted within the CXCL12 targeting allele. The previously generated CXCL12$^{loxPneo/+}$ non-chimeric mice were breed with Sox2-*Cre* hemizygous mice, resulting in the generation of CXCL12$^{+/-}$;Sox2-*Cre* mice. These CXCL12$^{+/-}$;Sox2-*Cre* progeny were then interbreed to generate neonatally lethal CXCL12$^{-/-}$;Sox2-*Cre* mice and neonatally viable CXCL12$^{+/-}$;Sox2-*Cre* and CXCL12$^{+/+}$;Sox2-*Cre* mice.

A.



B.

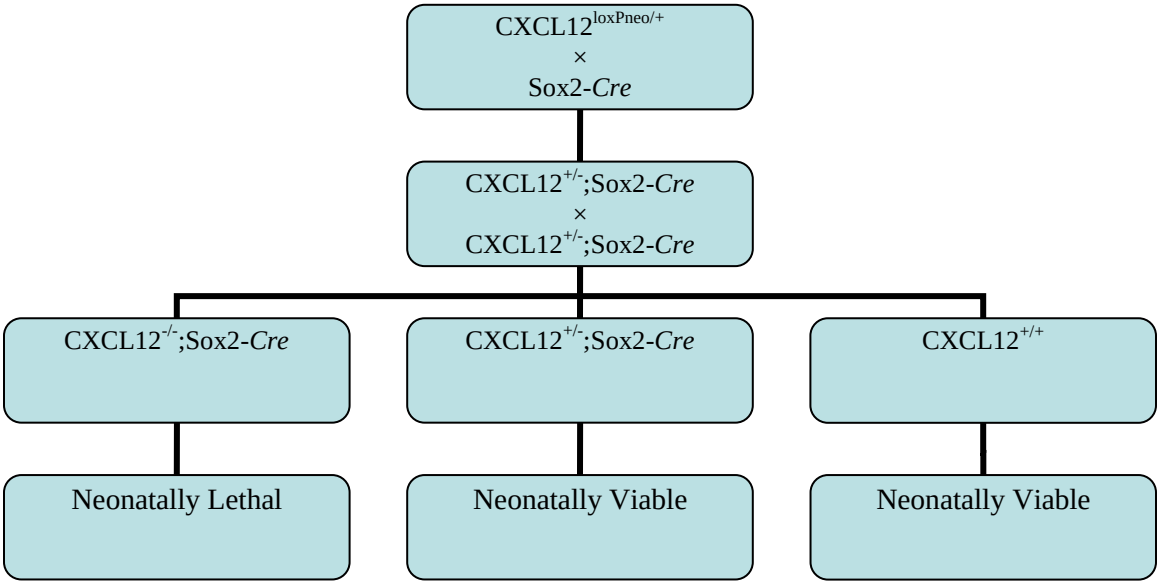


Figure 9. Sox2-*Cre*–Mediated Deletion of CXCL12. A. CXCL12 Targeting Allele. The $LoxP_1$ site is directly downstream of the 8.1 kbp long 5' arm, and the $LoxP_2$ site is located directly upstream of the 4.0 kbp short 3' arm. The introduction of *Cre* recombinase with Sox2-*Cre* mice will facilitate the deletion of the 7.0 kbp LoxP-flanked region, resulting in the deletion of the CXCL12 gene coding region and the deactivation of the CXCL12 gene throughout the early embryo. **B. CXCL12-Sox2-*Cre* Breeding Scheme**. This breeding scheme diagrams the generation of CXCL12$^{-/-}$;Sox2-*Cre* mice starting from CXCL12$^{loxPneo/+}$ mice. For the CXCL12 superscript, -/- denotes the deletion of the LoxP-flanked CXCL12 gene coding region in both alleles, +/- denotes the deletion of the LoxP-flanked CXCL12 gene coding region in one allele, and +/+ denotes the presence of the CXCL12 gene coding region in both alleles.

The interbreeding of CXCL12$^{LoxPneo/-}$;Sox2-*Cre* mice resulted in 18 total progeny. Of the 18 progeny, 5 (28%) were neonatally lethal and died within hours after birth, while 13 (72%) were neonatally viable.

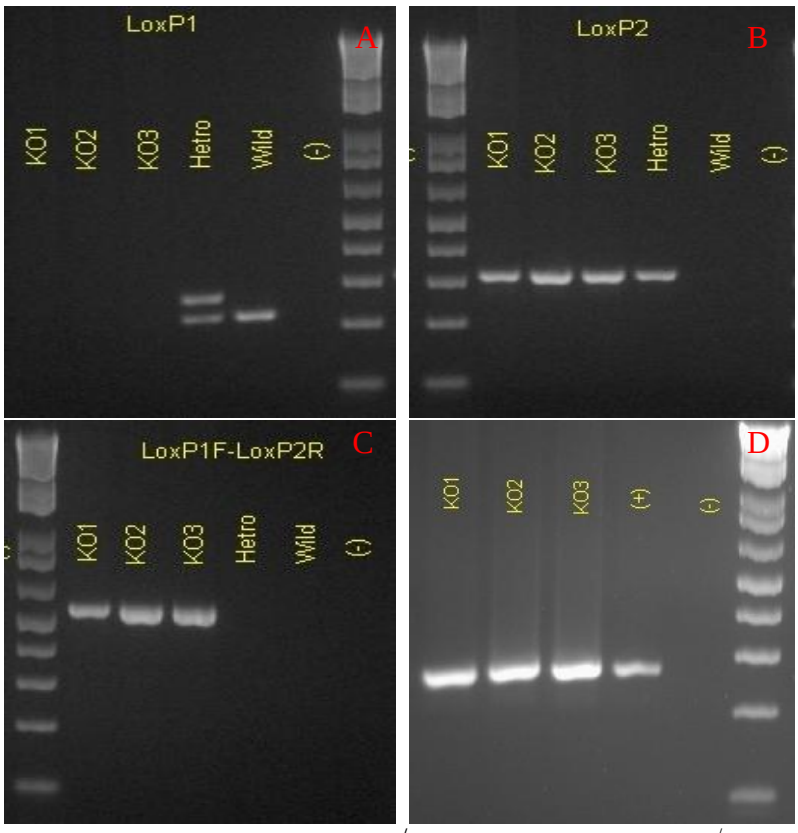


Figure 10. Genetic Characterization of CXCL12$^{-/-}$;Sox2-*Cre* Mice. CXCL12$^{-/-}$;Sox2-*Cre* Mice were genetically characterized for **A**. LoxP$_1$, **B.** LoxP$_2$, **C**. LoxP$_1$/LoxP$_2$, and **D.** Sox2-*Cre* using PCR assays with gene specific primers.

Table 2-A	
LoxP$_1$ Genotype	**Banding Pattern**
Homozygous	261 bp
Heterozygous	261 bp and 227 bp
Wildtype	227 bp

Table 2-B	
LoxP$_2$ Genotype	**Banding Pattern**
Homozygous	335 bp
Heterozygous	335 bp
Wildtype	No Band

Table 2-C	
LoxP$_1$LoxP$_2$ Genotype	**Banding Pattern**
Homozygous	567 bp
Heterozygous	567 bp
Wildtype	No Band

Table 2-D	
Sox2-*Cre* Genotype	**Banding Pattern**
Homozygous	260 bp
Heterozygous	260 bp
Wildtype	No Band

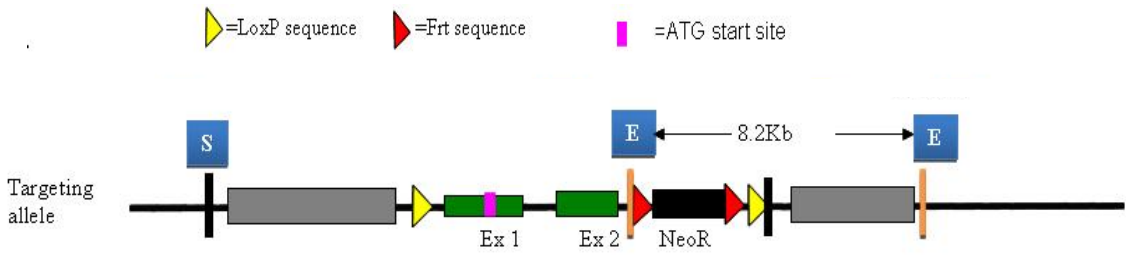
Table 2. CXCL12-Sox2-*Cre* PCR Product Banding Patterns. A. LoxP$_1$ Banding Pattern. **B.** LoxP$_2$ Banding Pattern. **C**. LoxP$_1$/LoxP$_2$ Banding Pattern. **D**.Sox2-*Cre* Banding Pattern.

As depicted in Figure 10, 3 of the 5 noenatally lethal CXCL12$^{-/-}$;Sox2-*Cre* mice were genetically characterized with a combination of 4 PCR assays: $LoxP_1$, $LoxP_2$, $LoxP_1$/$LoxP_2$, and Sox2-*Cre*. As expected, all 3 CXCL12$^{-/-}$;Sox2-*Cre* mice were $LoxP_1$-negative, $LoxP_2$-positive, $LoxP_1$/$LoxP_2$-positive, and Sox2-*Cre*-positive, confirming that the LoxP-flanked region of the CXCL12 targeting allele was excised due to the introduction of *Cre* recombinase with Sox2-*Cre* mice.

FLPeR-Mediated Deletion of Neo

A breeding scheme was created with the purpose of generating CXCL12$^{loxP/loxP,\ FLPeR\ (-/-)}$ mice (LoxP-homozygous, Neo-negative, FLPeR-negative), initially starting from the previously generated CXCL12$^{loxPneo/+}$ non-chimeric mice. These CXCL12$^{loxP/loxP,\ FLPeR\ (-/-)}$ mice will then be used in future breeding schemes to generate CXCL12 conditional knockout mice.

A.



B.

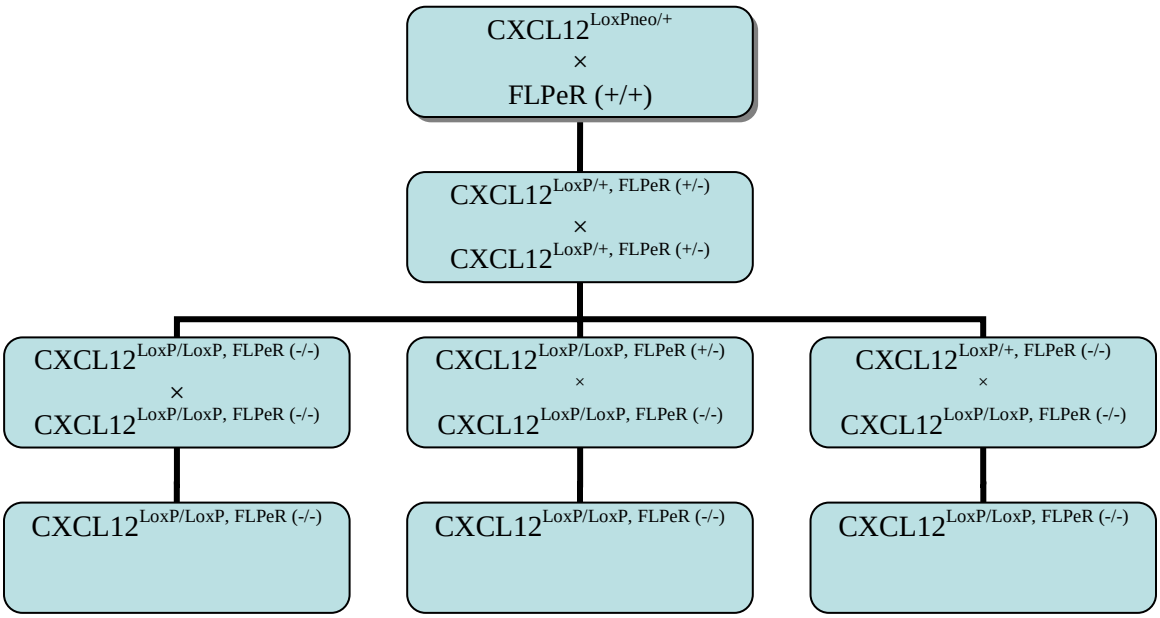


Figure 11. FLPeR-Mediated Deletion of Neo. A. CXCL12 Targeting Allele. The Frt_1 site is located directly upstream of the 1.8 kbp Neo coding region, and the Frt_2 site is located directly downstream of the Neo coding region, both located within the 7.0 kbp LoxP-flanked region. The introduction of FLPeR will facilitate the deletion of the Frt-flanked region, resulting in the removal of the neomycin resistance gene.
B. CXCL12-FLPeR Breeding Scheme Diagram. This breeding scheme diagrams the generation of CXCL12$^{LoxP/LoxP,\ FLPeR\ (-/-)}$ mice starting from CXCL12$^{LoxPneo/+}$ mice. For the CXCL12-LoxPneo superscript, LoxPneo/LoxPneo denotes a homozygous genotype, LoxPneo/+ denotes a heterozygous genotype, and +/+ denotes a wildtype genotype. For the CXCL12-LoxP superscript, LoxP/loxP denotes a homozygous genotype, LoxP/+ denotes a heterozygous genotype, and +/+ denotes a wildtype genotype. For the FLPeR superscript, FLPeR (+/+) denotes a homozygous genotype, FLPeR (+/-) denotes a heterozygous genotype, and FLPeR (-/-) denotes a wildtype genotype.

The previously generated $CXCL12^{LoxPneo/+}$ non-chimeric mice were breed with FLPeR-homozygous mice to remove the Frt-flanked Neo gene, resulting in the generation of $CXCL12^{LoxP/+,\ FLPeR\ (+/-)}$ mice. The $CXCL12^{LoxP/+,\ FLPeR\ (+/-)}$ mice were then interbreed to generate $CXCL12^{LoxP/LoxP,\ FLPeR\ (-/-)}$ mice with our target genotype. In order to generate a sufficiently large population of $CXCL12^{LoxP/LoxP,\ FLPeR\ (-/-)}$ mice, various combinations of $CXCL12^{LoxP/LoxP}$ and $CXCL12^{LoxP/+}$ mice were breed together to improve the chances of generating progeny with the $CXCL12^{LoxP/LoxP,\ FLPeR\ (-/-)}$ target genotype.

Generation of CXCL12$^{LoxP/LoxP,\ FLPeR\ (-/-)}$ Mice

CXCL12 Mice 3200-3210

Two CXCL12$^{LoxP/+,\ FLPeR\ (+/-)}$ mice were interbreed in order to generate CXCL12$^{LoxP/LoxP,\ FLPeR\ (-/-)}$ mice. This breeding yielded 11 progeny (3200-3210), which were then genetically characterized with a combination of 4 PCR assays: $LoxP_1$, $LoxP_2$, LoxP/Neo, and FLPeR.

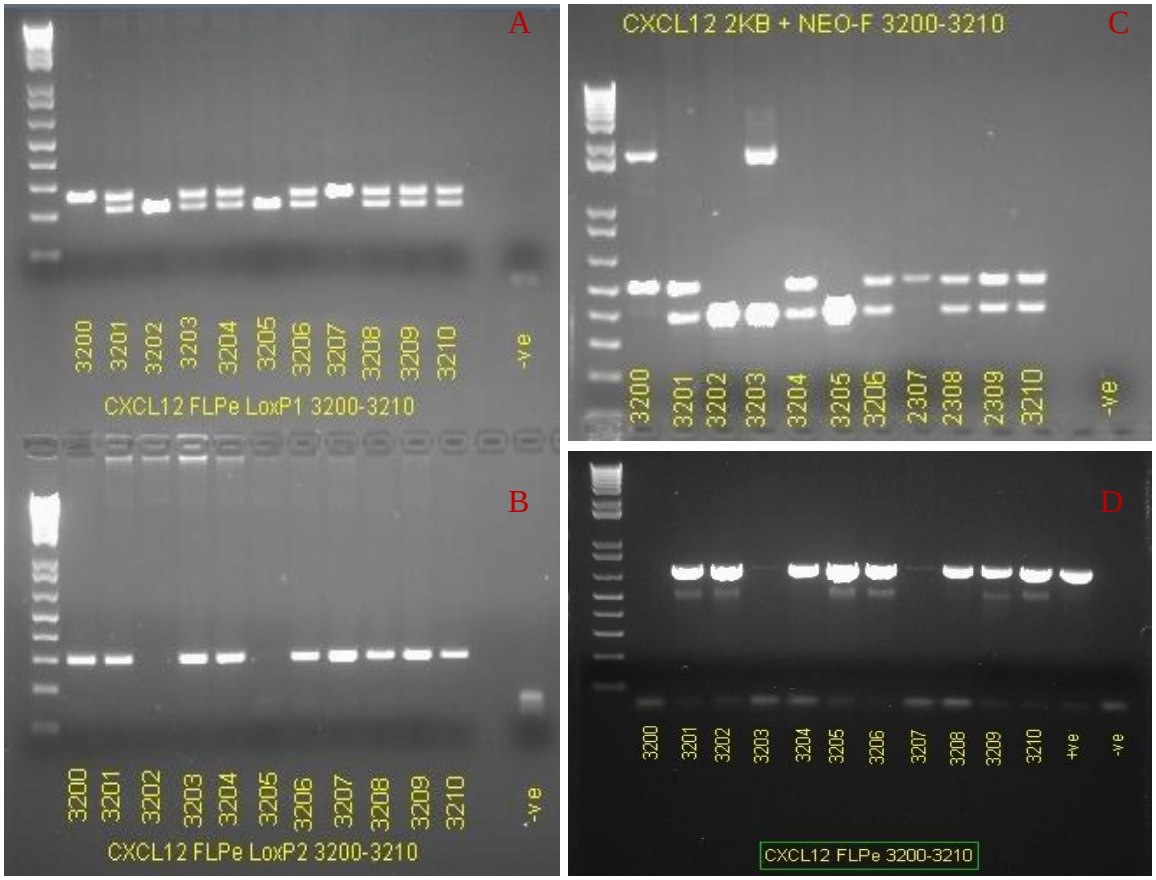


Figure 12. Genetic Characterization of CXCL12 Mice 3200-3210. CXCL12 mice 3200-3210 were genetically characterized for **A**. $LoxP_1$, **B**. $LoxP_2$, **C**. LoxP/Neo, and **D**. FLPeR using PCR assays with gene specific primers.

Table 3-A – $LoxP_1$	
$LoxP_1$ Genotype	**Band Size**
Homozygous	261 bp
Heterozygous	261 bp and 227 bp
Wild-Type	227 bp

Table 3-B – $LoxP_2$	
$LoxP_2$ Genotype	**Band Size**
Homozygous	335 bp
Heterozygous	335 bp
Wild-Type	No Band

Table 3-C – LoxP/Neo			
LoxP Genotype	**Band Size**	**Neo Genotype**	**Band Size**
Homozygous	515 bp	Homozygous	1.8 kbp
Heterozygous	515 bp and 319 bp	Heterozygous	1.8 kbp
Wildtype	319 bp	Wildtype	No Band

Table 3-D -FLPeR	
FLPeR Genotype	**Band Size**
Homozygous	725 bp
Heterozygous	725 bp
Wild-Type	No Band

Table 3. CXCL12-FLPeR PCR Product Banding Patterns. A. $LoxP_1$ Banding Pattern. **B.** $LoxP_2$ Banding Pattern. **C**. LoxP/Neo Banding Pattern. **D**. FLPeR Banding Pattern.

Sex/Tag #	$LoxP_1$	$LoxP_2$	FLPeR	Neo
F3200	+/+	+	-	+
M3201	+/-	+	+	-
M3202	-/-	-	+	-
M3203	+/-	+	-	+
M3204	+/-	+	+	-
F3205	-/-	-	+	-
F3206	+/-	+	+	-
F3207	+/+	+	-	-
M3208	+/-	+	+	-
M3209	+/-	+	+	-
M3210	+/-	+	+	-

Table 4. Genetic Characterization of CXCL12 Mice 3200-3210. For $LoxP_1$, +/+ denotes a homozygous genotype, +/- denotes a heterozygous genotype, and -/- denotes a wildtype genotype. For $LoxP_2$, Neo, and FLPeR, + denotes a homozygous or heterozygous genotype and – denotes a wildtype genotype.

This breeding yielded progeny with the following genotypes:

$CXCL12^{LoxP/LoxP,\ FLPeR\ (-/-)}$: F3207
$CXCL12^{LoxP/+,\ FLPeR\ (+)}$: M3201, M3204, F3206, M3208, 3209, 3210
$CXCL12^{+/+,\ FLPeR\ (+)}$: 3202, 3205
$CXCL12^{LoxPneo/LoxPneo,\ FLPeR\ (-/-)}$: F3200
$CXCL12^{LoxPneo/+,\ FLPeR\ (+)}$: 3203

This breeding pair yielded 1 mouse (3207) with the $CXCL12^{LoxP/LoxP,\ FLPeR\ (-/-)}$ target genotype, as well as 8 mice (3200, 3201, 3203, 3204, 3206, 3208, 3209, 3210) that can be used for breeding purposes in order to generate more mice with the target genotype.

Based on the results from the genetic characterization of CXCL12 mice 3200-3210, 3 breeding pairs were initiated on April 10, 2012:

1. F3207 ($CXCL12^{LoxP/LoxP,\ FLPeR\ (-/-)}$) × M3201 ($CXCL12^{LoxP/+,\ FLPeR\ (+)}$)
2. F3200 ($CXCL12^{LoxPneo/LoxPneo,\ FLPeR\ (-/-)}$) × M3208 ($CXCL12^{LoxP/+,\ FLPeR\ (+)}$)
3. F3206 ($CXCL12^{LoxP/+,\ FLPeR\ (+)}$) × M3204 ($CXCL12^{LoxP/+,\ FLPeR\ (+)}$)

CXCL12 Mice 3243-3252

Two $CXCL12^{LoxP/+,\ FLPeR\ (+/-)}$ mice were interbreed, resulting in 10 progeny (3243-3252). The progeny were genetically characterized using a combination of 2 PCR assays: LoxP/Neo and FLPeR.

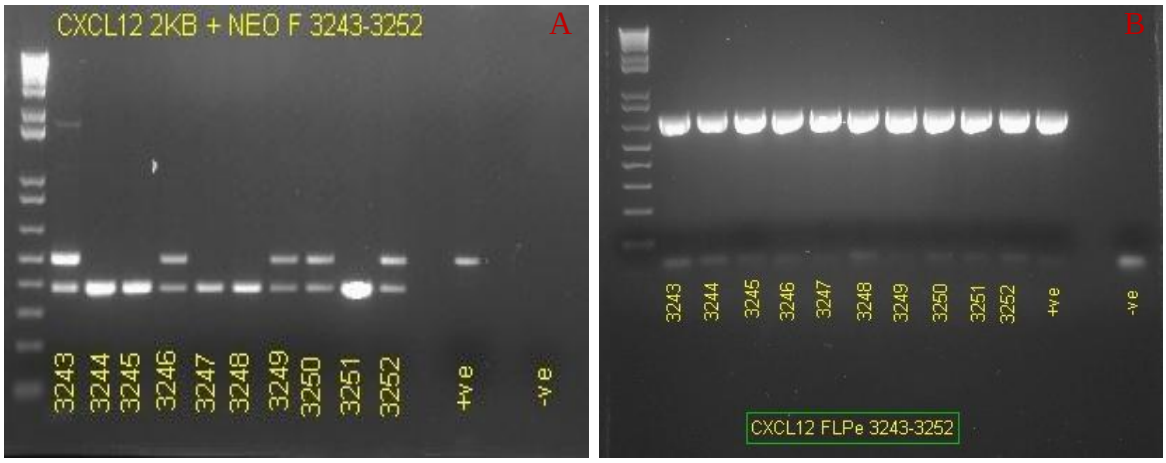


Figure 13. Genetic Characterization of CXCL12 Mice 3243-3252. CXCL12 mice 3243-3252 were genetically characterized for **A**. LoxP/Neo and **B**. FLPeR using PCR assays with gene specific primers.

Sex/Tag #	$LoxP_1$	$LoxP_2$	FLPeR	Neo
F3243	+/-	+	+	+
F3244	-/-	-	+	-
F3245	-/-	-	+	-
F3246	+/-	+	+	-
M3247	-/-	-	+	-
M3248	-/-	-	+	-
M3249	+/-	+	+	-
M3250	+/-	+	+	-
M3251	-/-	-	+	-
M3252	+/-	+	+	-

Table 5. Genetic Characterization of CXCL12 Mice 3243-3252. For $LoxP_1$, +/+ denotes a homozygous genotype, +/- denotes a heterozygous genotype, and -/- denotes a wildtype genotype. For $LoxP_2$, FLPeR, and Neo, + denotes a homozygous or heterozygous genotype and – denotes a wildtype genotype.

This breeding yielded progeny with the following genotypes:

$CXCL12^{LoxP/+,\ FLPeR\ (+)}$: 3246, 3249, 3250, 3252
$CXCL12^{LoxPneo/+,\ FLPeR\ (+)}$: 3243
$CXCL12^{+/+,\ FLPeR\ (+)}$: 3244, 3245, 3247, 3248, 3251

This breeding pair yielded no mice with the $CXCL12^{LoxP/LoxP,\ FLPeR\ (-/-)}$ target genotype, but yielded 4 mice (3246, 3249, 3250, 3252) that can be used for breeding purposes in order to generate more mice with the target genotype.

CXCL12 Mice 3268-3277

Two $CXCL12^{LoxP/+,\ FLPeR\ (+/-)}$ mice were interbreed, resulting in 10 progeny (3268-3277). The progeny were genetically characterized using a combination of 2 PCR assays: LoxP/Neo and FLPeR.

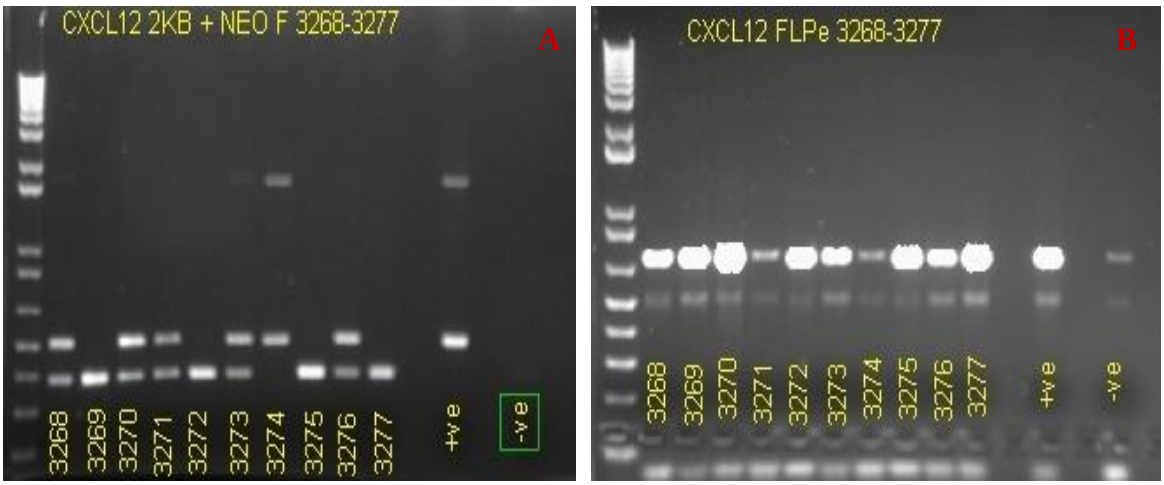


Figure 14. Genetic Characterization of CXCL12 Mice 3268-3277. CXCL12 mice 3268-3277 were genetically characterized for **A**. LoxP/Neo and **B**. FLPeR using PCR assays with gene specific primers.
Note: The bottom rung of the DNA ladder in the LoxP/Neo gel image ran off the bottom of the agarose gel.

Sex/Tag #	$LoxP_1$	$LoxP_2$	FLPeR	Neo
F3268	+/-	+	+	-
F3269	-/-	-	+	-
F3270	+/-	+	+	-
F3271	+/-	+	-	-
F3272	-/-	-	+	-
F3273	+/-	+	+	+
F3274	+/+	+	-	+
M3275	-/-	-	+	-
M3276	+/-	+	+	-
M3277	-/-	-	+	-

Table 6. Genetic Characterization of CXCL12 Mice 3268-3277. For $LoxP_1$, +/+ denotes a homozygous genotype, +/- denotes a heterozygous genotype, and -/- denotes a wildtype genotype. For $LoxP_2$, FLPeR, and Neo, + denotes a homozygous or heterozygous genotype and – denotes a wildtype genotype.

This breeding yielded progeny with the following genotypes:

$CXCL12^{LoxP/+,\ FLPeR\ (-/-)}$: 3271
$CXCL12^{LoxP/+,\ FLPeR\ (+)}$: 3268, 3270, 3276
$CXCL12^{+/+,\ FLPeR\ (+)}$: 3269, 3272, 3275, 3277
$CXCL12^{LoxPneo/LoxPneo,\ FLPeR\ (-/-)}$: 3274
$CXCL12^{LoxPneo/+,\ FLPeR\ (+)}$: 3273

This breeding pair yielded no mice with the $CXCL12^{LoxP/LoxP,\ FLPeR\ (-/-)}$ target genotype, but yielded 5 mice (3268, 3270, 3271, 3274, 3276) that can be used for breeding purposes in order to generate more mice with the target genotype.

CXCL12 Mice 3317-3325

Two $CXCL12^{LoxP/+,\ FLPeR\ (+/-)}$ mice were interbreed, resulting in 9 progeny (3317-3325). The progeny were genetically characterized using a combination of 2 PCR assays: LoxP/Neo and FLPeR.

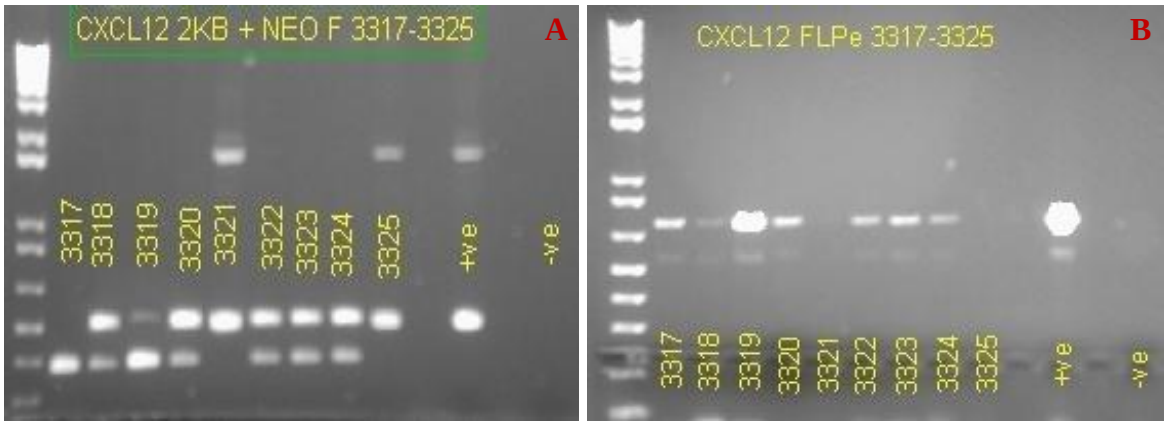


Figure 15. Genetic Characterization of CXCL12 Mice 3317-3325. CXCL12 mice 3317-3325 were genetically characterized for **A.** LoxP/Neo and **B.** FLPeR using PCR assays with gene specific primers. Note: The bottom 2 rungs of the DNA ladder in the LoxP/Neo gel image ran off the bottom of the gel.

Sex/Tag #	$LoxP_1$	$LoxP_2$	FLPeR	Neo
F3317	-/-	-	+	-
F3318	+/-	+	+	-
F3319	+/-	+	+	-
F3320	+/-	+	+	-
F3321	+/+	+	-	+
M3322	+/-	+	+	-
M3323	+/-	+	+	-
M3324	+/-	+	+	-
M3325	+/+	+	-	+

Table 7. Genetic Characterization of CXCL12 Mice 3317-3325. For $LoxP_1$, +/+ denotes a homozygous genotype, +/- denotes a heterozygous genotype, and -/- denotes a wildtype genotype. For $LoxP_2$, FLPeR, and Neo, + denotes a homozygous or heterozygous genotype and – denotes a wildtype genotype.

This breeding yielded progeny with the following genotypes:

$CXCL12^{LoxP/+,\ FLPeR\ (+)}$: 3318, 3319, 3320, 3322, 3323, 3324
$CXCL12^{+/+,\ FLPeR\ (+)}$: 3317
$CXCL12^{LoxPneo/LoxPneo,\ FLPeR\ (-/-)}$: 3321, 3325

This breeding pair yielded no mice with the $CXCL12^{LoxP/LoxP,\ FLPeR\ (-/-)}$ target genotype, but yielded 8 mice (3318, 3319, 3320, 3321, 3322, 3323, 3324, 3325) that can be used for breeding purposes in order to generate more mice with the target genotype.

Discussion

Sox2-*Cre*–Mediated Deletion of CXCL12

The breeding of Sox2-*Cre* mice with $CXCL12^{LoxPneo/+}$ non-chimeric mice resulted in the generation of $CXCL12^{+/-}$;Sox2-*Cre* progeny. The introduction of *Cre* recombinase resulted in the deletion of the LoxP-flanked CXCL12 gene coding region in one allele throughout the early embryo. The $CXCL12^{+/-}$;Sox2-*Cre* mice were then interbreed, resulting in the generation of progeny with the following expected genotypic ratio:

25% $CXCL12^{-/-}$;Sox2-*Cre*
50% $CXCL12^{+/-}$;Sox2-*Cre*
25% $CXCL12^{+/+}$;Sox2-*Cre*

In progeny with a $CXCL12^{-/-}$;Sox2-*Cre* genotype, the introduction of *Cre* recombinase resulted in the deletion of the LoxP-flanked CXCL12 gene coding region in both alleles throughout the early embryo. The CXCL12 protein is essential for several processes during the embryonic stage of development, including the development of vasculature within the GI tract.[15] Since the CXCL12 gene encodes for an essential protein during the embryonic stage of development, any progeny with a $CXCL12^{-/-}$;Sox2-*Cre* genotype were expected to be neonatally lethal. Any progeny with a functional CXCL12 gene in at least one allele, including progeny with a $CXCL12^{+/-}$;Sox2-*Cre* or $CXCL12^{+/+}$;Sox2-*Cre* genotype, were expected to be neonatally viable.

The interbreeding of $CXCL12^{+/-}$;Sox2-*Cre* mice yielded a total of 18 progeny. Of the 18 progeny, 5 (28%) were deceased within hours of birth, while 13 (72%) survived. The ratio of neonatally lethal and neonatally viable progeny closely approximated the expected ratio (25% lethal, 75% viable). Of the 5 neonatally lethal progeny, 3 were genetically characterized with PCR. As depicted in Figure 10, all 3 neonatally lethal mice were $CXCL12^{-/-}$;Sox2-*Cre* ($LoxP_1$-negative, $LoxP_2$-positive, $LoxP_1$/$LoxP_2$-positive, and Sox2-*Cre*-positive); the genotype expected in mice that have undergone the Sox2-*Cre*-mediated deletion of the CXCL12 gene.

The neonatal lethality of $CXCL12^{-/-}$;Sox2-*Cre* mice confirms that the CXCL12 targeting vector correctly inserted LoxP sequences both upstream of exon 1 and downstream of

exon 2 in the CXCL12 targeting allele. If the LoxP sequences were not flanking the CXCL12 gene coding region, then the introduction of *Cre* recombinase with Sox2-*Cre* mice would not have mediated the deletion of the CXCL12 gene and would therefore not have resulted in neonatal lethality in $CXCL12^{-/-}$;Sox2-*Cre* mice.

FLPeR-Mediated Deletion of Neo

The neomycin resistance gene was present within the CXCL12 targeting vector so that the successfully transfected ES cells could be selected for via neomycin treatment during the generation of $CXCL12^{LoxPneo/+}$ chimeric mice. Since the Neo gene is located downstream of the second exon in the CXCL12 gene, within the LoxP-flanked gene coding region of the CXCL12 targeting allele, it has the potential to affect the transcription of the CXCL12 gene, the translation of the CXCL12 gene produce, and therefore the function of the CXCL12 protein. For this reason, the Neo gene was removed from the CXCL12 targeting allele via the introduction of the flippase reporter gene using FLPeR mice.

A breeding scheme was constructed to generate $CXCL12^{LoxP/LoxP,\ FLPeR\ (-/-)}$ mice without the Neo gene, starting from the previously generated $CXCL12^{LoxPneo/+}$ non-chimeric mice. The $CXCL12^{LoxPneo/+}$ mice were breed with FLPeR mice, resulting in a hypomorphic mutation in the F_1 generation that removed the Frt-flanked Neo gene from the CXCL12 targeting allele, resulting in the generation of The $CXCL12^{LoxP/+,\ FLPeR\ (+/-)}$ mice. The $CXCL12^{LoxP/+,\ FLPeR\ (+/-)}$ hypomorphic mice were then interbreed to generate $CXCL12^{LoxP/LoxP,\ FLPeR\ (-/-)}$ mice; LoxP-homozygous, FLPeR-wildtype, hypomorphic mice with the Neo gene removed from both CXCL12 alleles. Several combinations of $CXCL12^{LoxP/LoxP,\ FLPeR\ (-/-)}$, $CXCL12^{LoxP/+,\ FLPeR\ (-/-)}$, and $CXCL12^{LoxP/LoxP,\ FLPeR\ (+/-)}$ mice were interbreed to generate more mice with our target genotype.

Generation of $CXCL12^{LoxP/LoxP,\ FLPeR\ (-/-)}$ Mice

As expected, the genetic characterization of CXCL12 –FLPeR mice 3200-3210 indicated that the $LoxP_1$ banding pattern observed in Figure 12-A mirrors the LoxP/Neo banding pattern observed in Figure 12-C. This observation justifies the use of 2 PCR assays (LoxP/Neo and FLPeR) to fully characterize a set of CXCL12-FLPeR mice, instead of 4

PCR assays ($LoxP_1$, $LoxP_2$, FLPeR, and LoxP/Neo), allowing for a more time-efficient genetic characterization process. Two PCR assays were used to genetically characterize all subsequent CXCL12-FLPeR mice following the genetic characterization of CXCL12-FLPeR mice 3200-3210. Also as expected, the $LoxP_1$ banding pattern observed in Figure 12-A is predictive of the $LoxP_2$ banding pattern observed in Figure 12-B. $LoxP_1$-homozygous and $LoxP_1$-heterozygous mice were all $LoxP_2$-positive, while $LoxP_1$-wildtype mice were all $LoxP_2$-negative. This observation indicates that the germline transmission of the CXCL12 targeting vector was successful, since $LoxP_1$ and $LoxP_2$ were both detected in concert.

After genetically characterizing CXCL12 mice 3200-3210 with PCR, 3 breeding pairs were initiated using mice from this group. The most important breeding pair was the breeding of F3207, the only mouse characterized with the $CXCL12^{loxP/loxP,\ FLPeR\ (-/-)}$ target genotype, with M3201, a $CXCL12^{LoxP/+,\ FLPeR\ (+)}$ mouse. Assuming that LoxP and FLPeR are passed on via a traditional Mendelian Inheritance pattern, there are two possible scenarios regarding the expected genotypic ratios of the progeny:

Possibility 1:
If M3201 is $CXCL12^{LoxP/+,\ FLPeR\ (+/-)}$ the progeny's genotypic ratio is expected to be:
25% $CXCL12^{loxP/loxP,\ FLPeR\ (-/-)}$ (Target Genotype)
25% $CXCL12^{loxP/+,\ FLPeR\ (-/-)}$
25% $CXCL12^{loxP/loxP,\ FLPeR\ (+/-)}$
25% $CXCL12^{loxP/+,\ FLPeR\ (+/-)}$

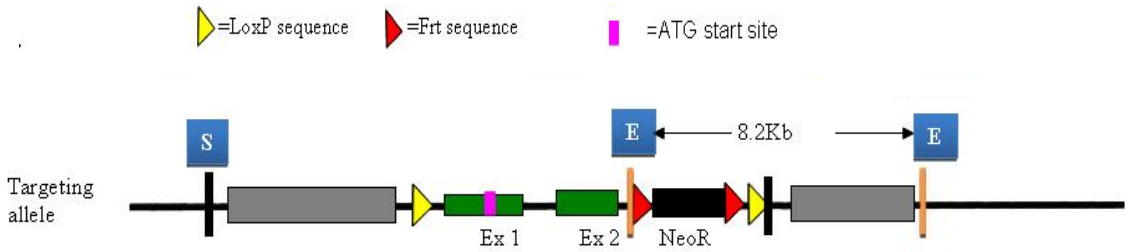
Possibility 2:
If M3201 is $CXCL12^{LoxP/+,\ FLPeR\ (+/+)}$, the progeny's genotypic ratio is expected to be:
50% $CXCL12^{loxP/loxP,\ FLPeR\ (+/-)}$
50% $CXCL12^{loxP/+,\ FLPeR\ (+/-)}$

If CXCL12 mouse 3201 is $CXCL12^{LoxP/+,\ FLPeR\ (+/-)}$, 25% of the progeny are expected to have the $CXCL12^{loxP/loxP,\ FLPeR\ (-/-)}$ target genotype. If the progeny includes both a male and a female mouse with the $CXCL12^{loxP/loxP,\ FLPeR\ (-/-)}$ target genotype, they can be breed together to generate progeny that are all expected to have the target genotype. Any progeny with the $CXCL12^{loxP/loxP,\ FLPeR\ (-/-)}$ target genotype can then be used in future breeding schemes.

Future Studies

It is hypothesized that the ablation of CXCL12 from stromal fibroblasts will inhibit tumor angiogenesis and progression in breast cancer by modulating the tumor microenvironment. In future studies, this hypothesis will be tested by generating CXCL12 conditional knockout mice from the $CXCL12^{LoxP/LoxP,\ FLPeR\ (-/-)}$ mice generated during this research project.

A.



B.

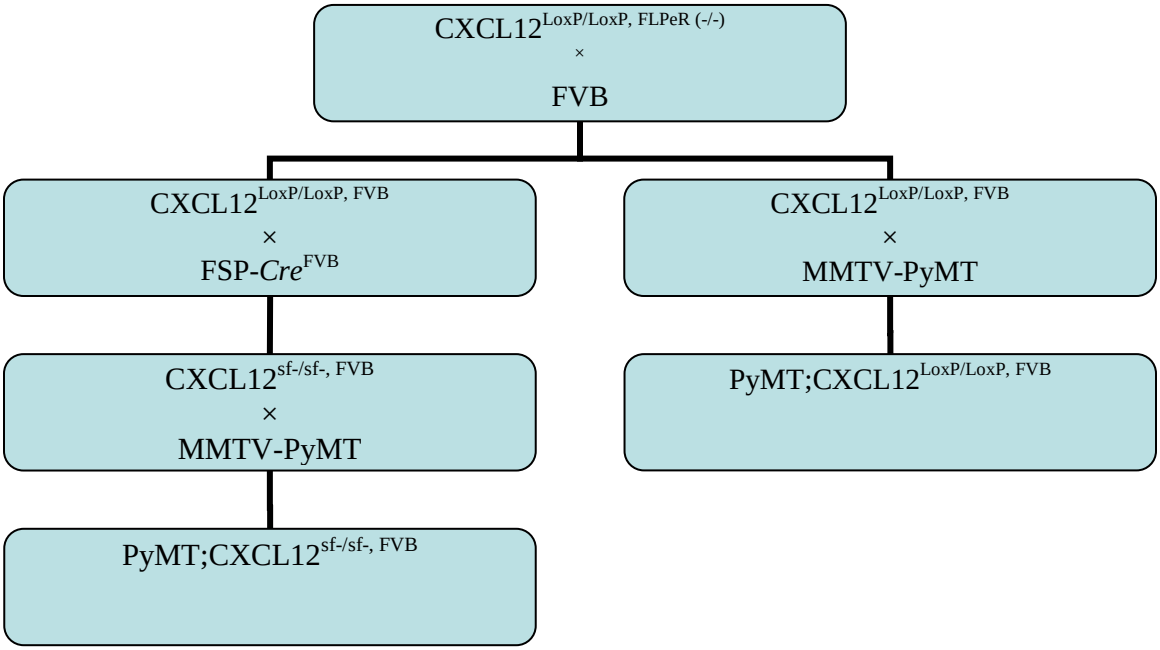


Figure 16. FSP-*Cre*-Mediated Generation of CXCL12 Conditional Knockout Mice A. CXCL12 Targeting Allele. The $LoxP_1$ site is directly downstream of the 8.1 kbp long 5' arm, and the $LoxP_2$ site is located directly upstream of the 4.0 kbp short 3' arm. The introduction of *Cre* recombinase with FSP-*Cre* mice will facilitate the deletion of the 7.0 kbp LoxP-flanked region, resulting in the deletion of the CXCL12 gene coding region and the deactivation of the CXCL12 gene from stromal fibroblasts in a cell/tissue type specific manner. **B. CXCL12-FSP-*Cre* Breeding Scheme Diagram**. This breeding scheme diagrams the generation of $PyMT;CXCL12^{sf-/sf-,\ FVB}$ and $PyMT;CXCL12^{LoxP/LoxP,\ FVB}$ mice starting from $CXCL12^{LoxP/LoxP,\ FLPeR\ (-/-)}$ mice. The FVB superscript denotes an FVB background. For the CXCL12 superscript, sf-/sf- denotes the deletion of the CXCL12 gene from stromal fibroblasts in both alleles, while LoxP/sf- denotes the deletion of CXCL12 gene from stromal fibroblasts in one allele.

In order to generate CXCL12 conditional knockout mice, $CXCL12^{loxP/loxP,\ FLPeR\ (-/-)}$ mice will initially be breed with FVB wildtype mice, resulting in the generation of $CXCL12^{loxP/loxP,\ FVB}$ mice. After five generations of CXCL12-FVB breeding, $CXCL12^{loxP/loxP,\ FVB}$ male mice will be breed with FSP-Cre^{FVB} female mice to generate $CXCL12^{sf-/sf-,\ FVB}$ progeny. The FSP-*Cre*-mediated deletion of the LoxP-flanked region from the CXCL12 targeting allele will result in the ablation of CXCL12 from stromal fibroblasts in a cell/tissue-type specific manner, resulting in the generation of CXCL12 conditional knockout mice. Since CXCL12 will only be deleted in stromal fibroblasts, the $CXCL12^{sf-/sf-,\ FVB}$ mice will still be neonatally viable. The neonatal viability of these CXCL12 conditional knockout mice allows them to sufficiently serve as models for the characterization of breast cancer growth and metastasis.

The $CXCL12^{sf-/sf-,\ FVB}$ mice will then be breed with Murine Mammary Tumor Virus-Polyoma Middle T-Antigen (MMTV-PyMT) transgenic mice to generate PyMT; $CXCL12^{sf-/sf-,\ FVB}$ mice, since the PyMT oncogene initiates the rapid onset and progression of mammary tumors. To determine the effects of the ablation of CXCL12 from stromal fibroblasts on breast cancer metastasis, the tumor growth and progression of PyMT;$CXCL12^{sf-/sf-,\ FVB}$ mice and PyMT;$CXCL12^{loxP/loxP,\ FVB}$ control mice will be observed over a 2-6 month period. After 2, 3, 4, and 6 months, mammary glands from both the PyMT; $CXCL12^{sf-/sf-,\ FVB}$ mice and the PyMT;$CXCL12^{loxP/loxP,\ FVB}$ control mice will be analyzed for tumor growth by whole mount carmine, H&E Staining, proliferation (Ki67, cyclin D1), macrophage (F4/80), and angiogenesis (CD31).

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